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Remarks

Applicants appreciate the allowance of claims 28-31, 51-55, 59, and 60.

The Rejection of Claims 32 and 56-58 Under 35 U.S.C. § 112, first paragraph

Claims 32 and 56-58 stand rejected under 35 U.S.C. § 112, first paragraph as failing to comply with the written description requirement. Applicants respectfully traverse the rejection.

The first paragraph of 35 U.S.C. § 112 requires that the specification provide a written description of the claimed invention:

[t]he specification shall contain a written description of the invention, and of the manner and process of making and using it, in such full, clear, concise, and exact terms as to enable any person skilled in the art to which it pertains, or with which it is most nearly connected, to make and use the same, and shall set forth the best mode contemplated by the inventor of carrying out his invention.

The purpose of the written description requirement is to ensure that the specification conveys to those skilled in the art that the applicants possessed the claimed subject matter as of the filing date sought. *Vas-Cath Inc. v. Mahurkar*, 935 F.2d 1555, 1563-64, 19 U.S.P.Q.2d (BNA) 1111, 1117 (Fed. Cir. 1991).

Claims 32 and 56-58 are directed to the composition of claim 28 “wherein a population of the molecular complexes is bound to the cell, wherein an identical antigenic peptide is bound to each ligand binding site.” The Office Action asserts that the specification does not provide an adequate description of the genus of antigenic peptides sufficient to show possession of the invention of claims 32 and 56-58. Office Action at pages 3-4.

“The description need only describe in detail that which is new or not conventional in the art.” M.P.E.P. § 2163(II)(A)(3)(a), citing *Hybritech v. Monoclonal Antibodies*, 802 F.2d 1367, 1384, 231 U.S.P.Q. (BNA) 81, 94 (Fed. Cir. 1986). Independent claim 28 is directed to a new and unconventional composition in which a molecular complex comprising four recited fusion proteins is bound to a cell. Claim 28 has been allowed; thus, the U.S. Patent and Trademark Office acknowledges that the specification adequately describes the new and unconventional composition of claim 28. *See also* the Office Action at page 2, item 4, which states that the written description supports the recited molecular complex.

The recited molecular complex contains two ligand binding sites. The specification teaches that the ligand binding sites can contain a bound ligand, “preferably an antigenic peptide.” Page 19, lines 8-9. In contrast to the molecular complex itself, antigenic peptides are neither new nor unconventional. It has long been known in the art that antigenic peptides are formed by the processing of internalized proteins in endosomal/lysosomal vesicles to peptides that can be presented by antigen presenting cells. *See Abbas et al., Cellular and Molecular Immunology*, 3rd ed., W.B. Saunders Company, Philadelphia, 1997, pages 125-37 (Attachment 1). Attachment 2 provides the results of two PubMed searches.¹ A PubMed search for “antigenic peptide” identified 848 references, dating back as far as 1979 (*e.g.*, Smith *et al.*, 1979, included in Attachment 2); a PubMed search for “peptide antigen” identified 517 references, dating back as far as 1965 (Akuzawa & Tsuchiya, 1965, Attachment 2). Because antigenic peptides are neither new nor unconventional, the specification need not describe antigenic

¹ The “PubMed” database includes bibliographic information from *inter alia* MEDLINE and OLDMEDLINE.

peptides in detail to support dependent claims to the composition of claim 28 "wherein an identical antigenic peptide is bound to each ligand binding site."

The Office Action cites *Regents of the University of California v. Lilly* to support the written description rejection. *Lilly* addressed what is required for a written description of novel genetic material. None of the rejected claims recites any genetic material, and the antigenic peptides are not *per se* novel. Thus, the Office Action's reliance on *Lilly* is misplaced.

The specification adequately describes the invention of dependent claims 32 and 56-68 because it describes the new and unconventional subject matter encompassed within those claims. Applicants respectfully request withdrawal of the rejection.

Respectfully submitted,
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April 19, 2004
Date

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CELLULAR AND MOLECULAR IMMUNOLOGY

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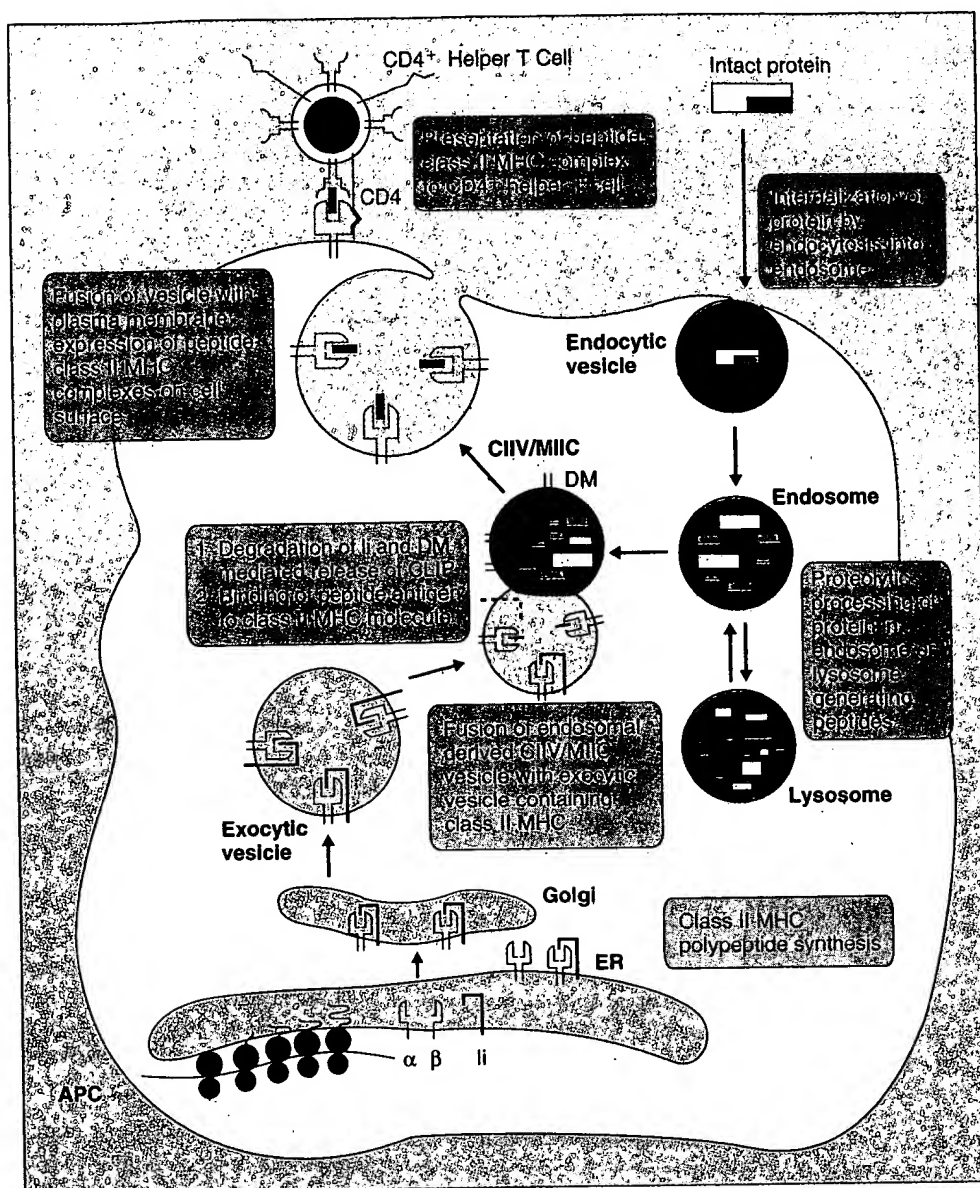


FIGURE 6-5. The class II major histocompatibility complex (MHC) pathway of antigen presentation. CLIP, class II-associated invariant chain peptide; Ii, invariant chain; ER, endoplasmic reticulum. Details of the functions of Ii and DM are shown in Figure 6-7.

that bind to class II MHC molecules, and most of these proteins are internalized from the extracellular environment. Thus, antigens made by extracellular bacteria, fungi, protozoa, and helminths are usually presented by the class II MHC pathway and activate CD4⁺ T cells. Additionally, some intact microorganisms can enter a cell by endocytosis or phagocytosis and survive within intracellular membrane-bound vesicles. Peptides derived from proteins made by these intracellular microorganisms may also be presented by class II MHC molecules.

Processing of Internalized Proteins in Endosomal/Lysosomal Vesicles

The next step in antigen presentation is the processing of the antigen that was internalized in its native form. Several characteristics of the pro-

cessing of extracellularly derived protein antigens are known:

1. *Antigen processing is a time- and metabolism-dependent phenomenon that takes place subsequent to internalization of antigen by APCs.* If macrophages (or other APCs) are incubated briefly ("pulsed") with a protein antigen such as ovalbumin, rendered metabolically inert by chemical fixation at various times thereafter, and tested for their ability to stimulate ovalbumin-specific T cells, functional antigen presentation occurs only if 1 to 3 hours elapse between the antigen pulse and fixation (Fig. 6-6). This time is required for the APCs to process the antigen and present it in association with class II MHC molecules on the cell surface. Processing of antigen is inhibited by maintaining the APCs below physiologic temperatures, by adding metabolic inhibitors such as azide, or by fixation earlier than 1 hour after the antigen pulse.

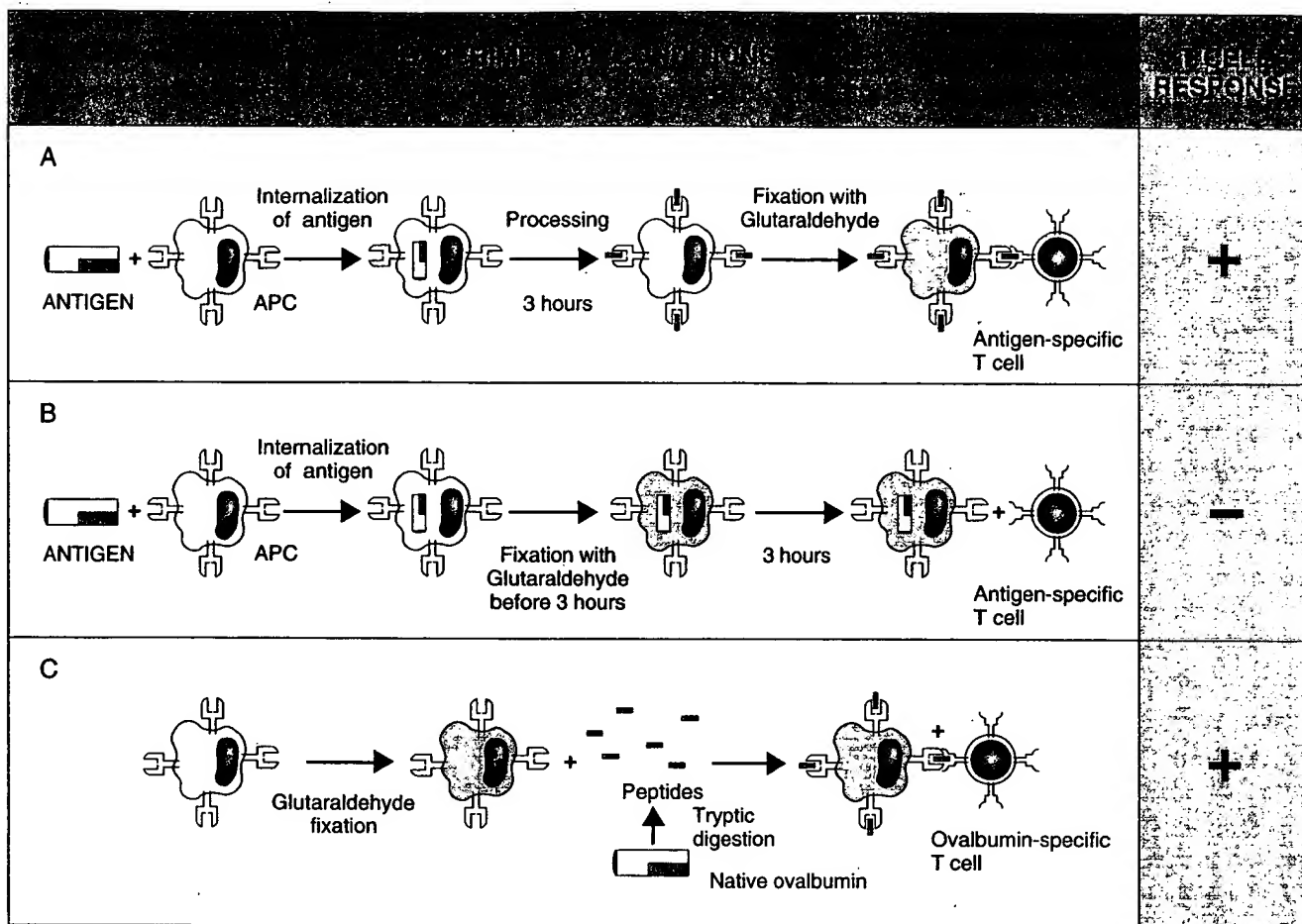


FIGURE 6-6. Antigen processing requires time and cellular metabolism and can be mimicked by *in vitro* proteolysis. If an antigen-presenting cell (APC) is allowed to process antigen and is then chemically fixed (rendered metabolically inert) 3 hours or more after antigen internalization, it is capable of presenting antigen to T cells (A). Antigen is not processed or presented if APCs are fixed less than 1 to 3 hours after antigen uptake (B). Fixed APCs bind and present proteolytic fragments of antigens to specific T cells (C). The artificial proteolysis, therefore, mimics physiologic antigen processing by APCs. Effective antigen presentation is assayed by measuring a T cell response, such as cytokine secretion. (Note that T cell hybridomas respond to processed antigens on fixed APCs, but growth factor-dependent T cells may require costimulators that are destroyed by fixation.)

2. *The endosomes and lysosomes where antigen processing takes place have an acidic pH, which is required for the processing.* Chemical agents that increase the pH of intracellular acid vesicles, such as chloroquine and ammonium chloride, are potent inhibitors of antigen processing.

3. *Cellular proteases are required for the processing of many protein antigens.* Several types of proteases, including cathepsin and leupeptin, are present in endosomes and lysosomes, and specific inhibitors of these enzymes block the presentation of protein antigens by APCs. The function of proteases is to cleave native protein antigens into small peptides. These proteases also probably act on the invariant chain, promoting its dissociation from class II MHC molecules, as discussed later. Most of these proteases function optimally at acid pH, and this is the likely reason why antigen processing occurs best in acidic compartments.

The processed forms of most protein antigens that T cells recognize can be artificially generated

by proteolysis in the test tube. Macrophages that are fixed or that are treated with chloroquine before exposure to antigen can effectively present pre-digested peptide fragments of that antigen, but not the intact protein, to specific T cells (Fig. 6-6). Peptides that bind to MHC molecules and stimulate T cells can be analyzed for amino acid sequence and secondary structure to determine the nature of the potential ligands for T cell antigen receptors. Immunogenic peptides derived from many complex globular proteins, such as cytochrome c, ovalbumin, myoglobin, and lysozyme, have been characterized in detail in this way. More recently, naturally generated peptides have been eluted from the class II MHC molecules of APCs and analyzed for common structural characteristics. The physicochemical features of peptides that permit their binding to MHC molecules were described in Chapter 5.

The net result of processing of a protein antigen is the generation of peptides, many of which are 10 to 30 amino acids long and capable of bind-

ing to the peptide-binding clefts of class II MHC molecules. The requirement for antigen processing prior to T cell stimulation explains why T cells recognize linear but not conformational determinants of proteins and why T cells cannot distinguish between native and denatured forms of a protein antigen (see Table 6-1). It is likely that most types of APCs, including macrophages, B cells, and dendritic cells, are qualitatively similar in their ability to process endocytosed antigens; however, there may be quantitative differences. For instance, macrophages contain many more proteases than do B cells and are more actively phagocytic, so that macrophages may be more efficient than B cells at internalizing and processing large particulate antigens and presenting peptide fragments of these antigens. It is also possible that different APCs generate distinct sets of peptides from the same native protein because of differences in their endosomal proteases. Furthermore, different APCs may present different peptides because the set of class II MHC molecules expressed by one APC may not be identical to those expressed by another. Therefore, it is possible that the APCs involved in presenting a particular protein antigen can influence which T cells are activated by that antigen.

Association of Processed Peptides With Newly Synthesized Class II MHC Molecules

Peptides generated by proteolysis of proteins in endosomes and lysosomes bind to newly synthesized class II MHC molecules within intracellular vesicles (see Fig. 6-5). The exact site of this association is not definitely known, but a variety of experimental data indicate that it occurs within an organelle of the endocytic pathway. An understanding of how peptide-class II MHC complexes are formed requires knowledge of the biosynthesis and subcellular transport of new class II MHC molecules. Several steps and key features of this process have been defined.

1. *The α and β chains of class II MHC molecules are coordinately synthesized and associate with each other in the endoplasmic reticulum (ER).* These chains are translated from messenger ribonucleic acid (mRNA) molecules on membrane-bound ribosomes and are co-translationally inserted into the membrane of the ER.

2. *Newly synthesized class II heterodimers temporarily associate with two other nonpolymorphic polypeptides, not encoded by the MHC, which are required for proper assembly and transport of the MHC molecule.* The first of these proteins is called **calnexin** and it functions as a molecular chaperone, ensuring that the α and β chains are properly folded during assembly of a class II MHC molecule. Calnexin is also involved in the assembly of other multichain molecules in the ER, including class I MHC molecules and the T cell antigen receptor

(see Chapter 7). The second nonpolymorphic protein associated with the class II MHC $\alpha\beta$ heterodimers in the ER is called the **invariant chain (Ii)**. This protein is a 30 kD Ig superfamily member which is a type II membrane protein, i.e., it has a reverse orientation to most transmembrane proteins, so that the amino terminus is intracytoplasmic and the carboxy terminus is intraluminal. The native invariant chain is a homotrimer. Each subunit binds one newly synthesized class II $\alpha\beta$ heterodimer, forming a nine polypeptide chain complex (i.e., three $\alpha\beta$ heterodimers bound to one invariant chain homotrimer). Only after the invariant chain binds the $\alpha\beta$ heterodimer is calnexin released, and the class II-invariant chain complex is able to move out of the ER.

3. *The invariant chain prevents peptides or nascent unfolded polypeptides in the ER from binding to newly formed class II MHC $\alpha\beta$ heterodimers.* The invariant chain binds to the class II MHC heterodimer in a way that interferes with peptide loading of the cleft formed by the α and β chains. There are, in fact, peptides within the ER derived from cytosolic proteins, as we will discuss later. Since the effector functions of class II-restricted T cells are best suited for dealing with extracellular microbes, it would be counterproductive to have class II MHC molecules loaded with peptides derived from cytosolic proteins. Furthermore, since the peptide binding cleft of class II MHC molecules has open ends, it can theoretically accommodate binding of newly translated polypeptides which have not yet folded into their tertiary structural conformation. Such polypeptides are abundant in the ER, but the presence of the invariant chain prevents their association with class II MHC molecules.

4. *The invariant chain also directs newly formed class II MHC molecules to specialized endosomal/lysosomal organelles where internalized proteins are proteolytically degraded into peptides.* In the ER, N-linked oligosaccharides are added to the newly translated class II MHC α and β chains, the two chains form heterodimers, and the heterodimers associate with invariant chains. Subsequent to these events, the class II MHC-invariant chain complexes pass through the Golgi apparatus, where the oligosaccharides are further modified. Then the invariant chain targets the movement of the mature class II MHC molecules to specialized membrane-bound organelles of the endocytic pathway that contain proteolytically degraded proteins derived from the extracellular milieu. The invariant chain performs this function by virtue of certain amino acid sequences in its amino terminal cytoplasmic tail. Immunoelectronmicroscopy and subcellular fractionation studies have been used to define specific characteristics of this subcellular compartment targeted by the invariant chain. In macrophages, it is called the MHC class II compartment or MHC and has the properties of a vesicle in transition between endosome and lysosome, in-

cluding high density and a characteristic multivesiculated appearance. In some B cells, a similar but less dense organelle containing invariant chain and class II MHC has been identified and named the class II vesicle (CIIV). These organelles likely represent specialized branch points in the vesicular transport pathways that allow newly formed class II MHC molecules on their way to the cell surface to become exposed to endocytically derived peptides. Thus, the invariant chain plays a key role in getting MHC molecules to the same place as peptides derived from extracellular protein antigens.

5. Within the MIIC/CIIV compartment, the invariant chain is removed from class II MHC molecules by the combined action of proteolytic enzymes and the HLA-DM molecule (see Fig. 6-7). Since the invariant chain blocks access to the peptide-binding groove of a class II MHC molecule, it must be removed before complexes of peptide and class II MHC can form. The same proteolytic enzymes that generate peptides from internalized proteins also act on the invariant chain in a stepwise fashion, leaving only a 24 amino acid remnant called class II-associated invariant chain peptide (CLIP). X-ray crystallographic analysis has shown that the CLIP peptide sits in the peptide-binding cleft in the same way that other peptides bind to class II MHC molecules. Therefore, removal of CLIP is required before ac-

cess is provided to peptides from extracellular proteins. This is accomplished by the action of a molecule called HLA-DM (or H-2M in the mouse), which is encoded within the MHC and has a structure very similar to that of class II MHC molecules. HLA-DM molecules differ from class II MHC molecules in several respects: they are not polymorphic, they do not necessarily associate with invariant chain, they are not expressed on the cell surface, and their subcellular distribution is distinct from class II MHC molecules. Nonetheless, HLA-DM is found in the MIIC compartment. Mutant cell lines which lack DM are defective in presenting peptides from extracellularly derived proteins. When class II MHC molecules are isolated from these DM-mutant cell lines, they are found to have almost exclusively CLIP peptides in their peptide-binding clefts, consistent with a role for DM in removing CLIP. *In vitro* studies have confirmed that HLA-DM acts as a peptide exchange molecule, facilitating the removal of CLIP and the addition of other peptides to class II MHC molecules. Predictably, DM gene knockout mice have profound defects in class II MHC-restricted antigen presentation.

6. Once CLIP peptides are removed, peptides generated by proteolytic cleavage of extracellularly derived protein antigens bind to class II MHC mole-

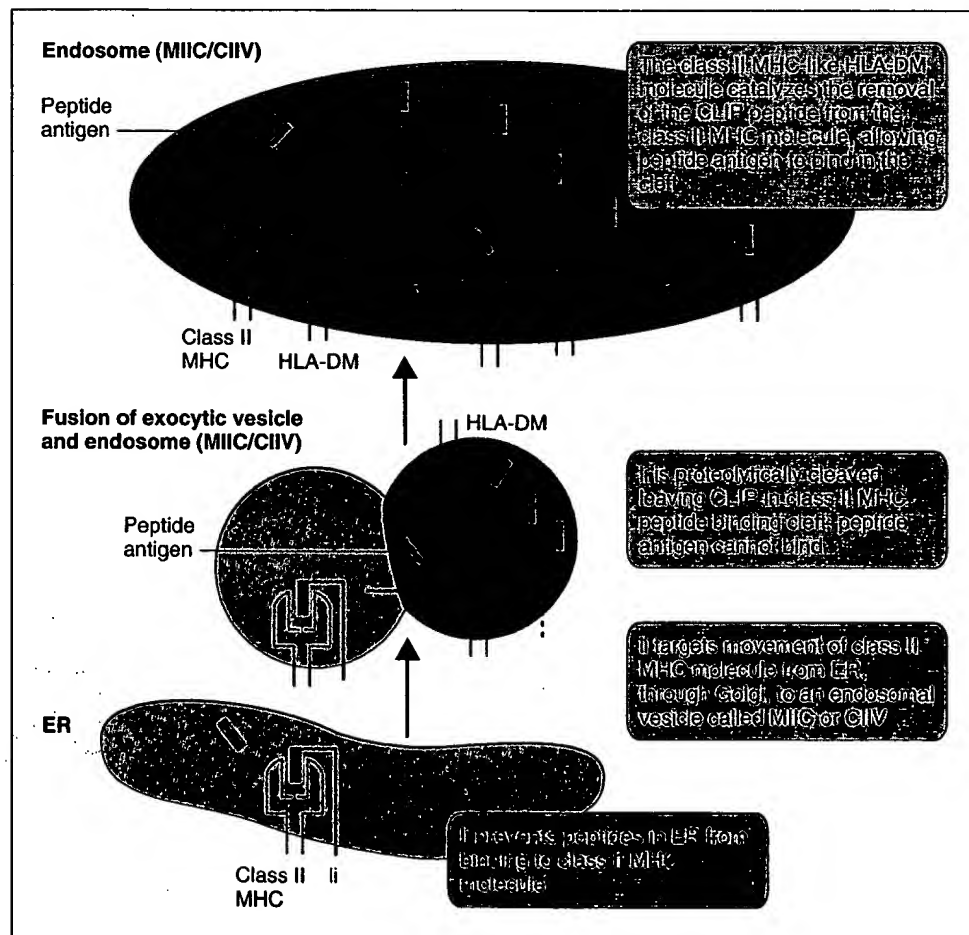


FIGURE 6-7. The functions of class II major histocompatibility complex (MHC)-associated invariant chains and HLA-DM. ER, endoplasmic reticulum; Ii, invariant chain.

cules. Although initial studies of the physical interaction of peptides with class II MHC molecules indicated a very slow association rate, requiring up to 48 hours to achieve saturation, more recent analyses indicate that HLA-DM greatly enhances this process, so that peptides can form stable complexes with class II MHC molecules within 20 minutes. Since the ends of the class II MHC peptide-binding cleft are open, large peptides or even unfolded whole proteins may bind, yet the size of peptides eluted from cell surface class II MHC molecules is restricted to between 10 and 30 amino acids. It is possible, therefore, that proteolytic enzymes "trim" bound polypeptides to the appropriate size for T cell recognition after the polypeptides bind to class II MHC molecules.

7. *Peptide binding to class II MHC molecules stabilizes the $\alpha\beta$ heterodimer, and the peptide dissociation rate is extremely slow.* The ability of peptide to increase the tightness of association of the class II MHC α and β chains serves to increase the likelihood that only properly loaded peptide-MHC complexes will survive long enough to get displayed on the cell surface. A similar phenomenon occurs in class I MHC assembly. The long life of a peptide-MHC complex increases the chance that a T cell specific for such a complex will make contact, bind, and be activated by that complex.

8. *Stable peptide-class II MHC complexes are delivered to the cell surface by membrane fusion with exocytic vesicles, and they are displayed there for surveillance by CD4⁺ T cells.*

Only a very small fraction of cell surface peptide-MHC complexes will contain the same peptide. Furthermore, most of the bound peptides will be derived from normal self proteins, since there is no mechanism to distinguish self from foreign proteins in the process that generates the peptide-MHC complexes. This has been demonstrated by amino acid analysis of peptides eluted from class II MHC molecules purified from B cells grown in tissue culture. Most of these peptides were derived from self proteins. These findings raise two important questions. First, if individuals process their own proteins and present them in association with their own class II MHC molecules, why do we normally not develop immune responses against self proteins? It is likely that self-tolerance is mainly due to the absence or inactivation of T cells capable of recognizing and responding to self antigens, and this is why self peptide-MHC complexes do not normally induce autoimmunity (see Chapters 8 and 19). Second, how can a T cell recognize and be activated by specific foreign antigen when it encounters an APC surface that is predominantly displaying self-peptide-MHC complexes? The answer lies in part with the extraordinary sensitivity of T cells for specific peptide-MHC complexes. It has been estimated that as few as 100 to 200 complexes of a particular peptide with a particular allelic form of class II MHC molecule on the surface of

an APC can lead to activation of a T cell. This represents less than 0.1 per cent of the total number of class II molecules likely to be present on the surface of the APC, most of which would be occupied with self peptides. In fact, the indiscriminate ability of the APC to internalize, process, and present the heterogeneous mix of self and foreign extracellular proteins ensures that the immune system will not miss transient or quantitatively small exposures to foreign antigens. Furthermore, there is evidence that a single T cell will sequentially engage multiple peptide-MHC complexes until achieving a sufficient threshold of activating signals (see Chapter 7).

Although the bulk of experimental evidence supports the model described above for the generation of most class II MHC-peptide complexes, there are potentially important alternate intracellular pathways for the generation of these complexes that may be immunologically significant. First, it is possible that cell surface class II molecules may be recycled by internalization into endosomes, where they bind newly generated peptide fragments of internalized protein. This process would likely require an exchange of previously bound peptides with the new ones. Second, there are exceptions to the general case that class II MHC molecules bind peptides derived from internalized exogenous proteins. Cell surface complexes of class II MHC molecules with peptides derived from endogenously synthesized proteins have been detected both by T cell responses to such proteins and by direct analysis of eluted peptides from cell surface-derived class II MHC molecules. In some cases, this may result from a normal cellular pathway for the turnover of cytoplasmic contents, referred to as autophagy. In this pathway, cytoplasmic contents are entrapped within ER-derived membrane vesicles called autophagosomes, these vesicles fuse with lysosomes, and the cytoplasmic proteins are proteolytically degraded. The association of the peptides generated by this route would require movement of the peptides to a class II-bearing vesicular compartment, as described previously for trafficking of exogenously derived peptides. In addition, some peptides that associate with class II MHC are derived from endogenously synthesized membrane proteins. Before they are expressed on the surface, these proteins may have ready access to class II MHC molecules because they would be synthesized and transported through the same ER-Golgi compartments as the membrane-bound class II MHC molecules themselves. How such membrane proteins are processed is currently unknown. It is also possible that after cell surface expression, membrane proteins may reenter the cell by the same endocytic pathway as exogenous proteins. In this way, peptides derived from virally encoded membrane proteins may enter the class II-MHC pathway of antigen presentation. This is a theoretically important way in which viral antigen-specific CD4⁺ helper T cells may be activated.

MECHANISMS OF ANTIGEN PRESENTATION TO CLASS I MHC-RESTRICTED CD8⁺ T CELLS

As we have mentioned previously, CD8⁺ T cells, most of which are CTLs, recognize peptides that are usually derived from protein antigens that are synthesized within APCs, processed, and subsequently expressed on the APC surface in association with class I MHC molecules. Examples of endogenously synthesized foreign proteins are viral proteins and the products of mutated or dysregulated genes in tumor cells. CTLs are the principal immunologic defense mechanisms against viruses and may be important in the immune destruction of tumors. In contrast to the restricted expression of class II MHC molecules, almost all cells express class I MHC molecules and have the ability to display peptide antigens in association with these MHC molecules on the cell surface. This ensures that any cell synthesizing viral or mutant proteins

can be marked for recognition and killing by CD8⁺ CTLs. As is the case with class II MHC-associated antigen presentation, generation of peptide-class I MHC complexes is a continuous normal function of cells, which does not discriminate between foreign and self proteins. This portion of the chapter describes the known features of the generation of peptide-class I MHC complexes on the surface of cells. The principal steps in this pathway are as follows (Fig. 6-8):

1. Synthesis of protein antigens in the cytosol or delivery of protein antigens into the cytosol
2. Proteolytic degradation of cytosolic proteins into peptides
3. Transport of peptides into the ER
4. Assembly of peptide-class I MHC complexes within the ER
5. Expression of peptide-class I MHC complexes on the cell surface

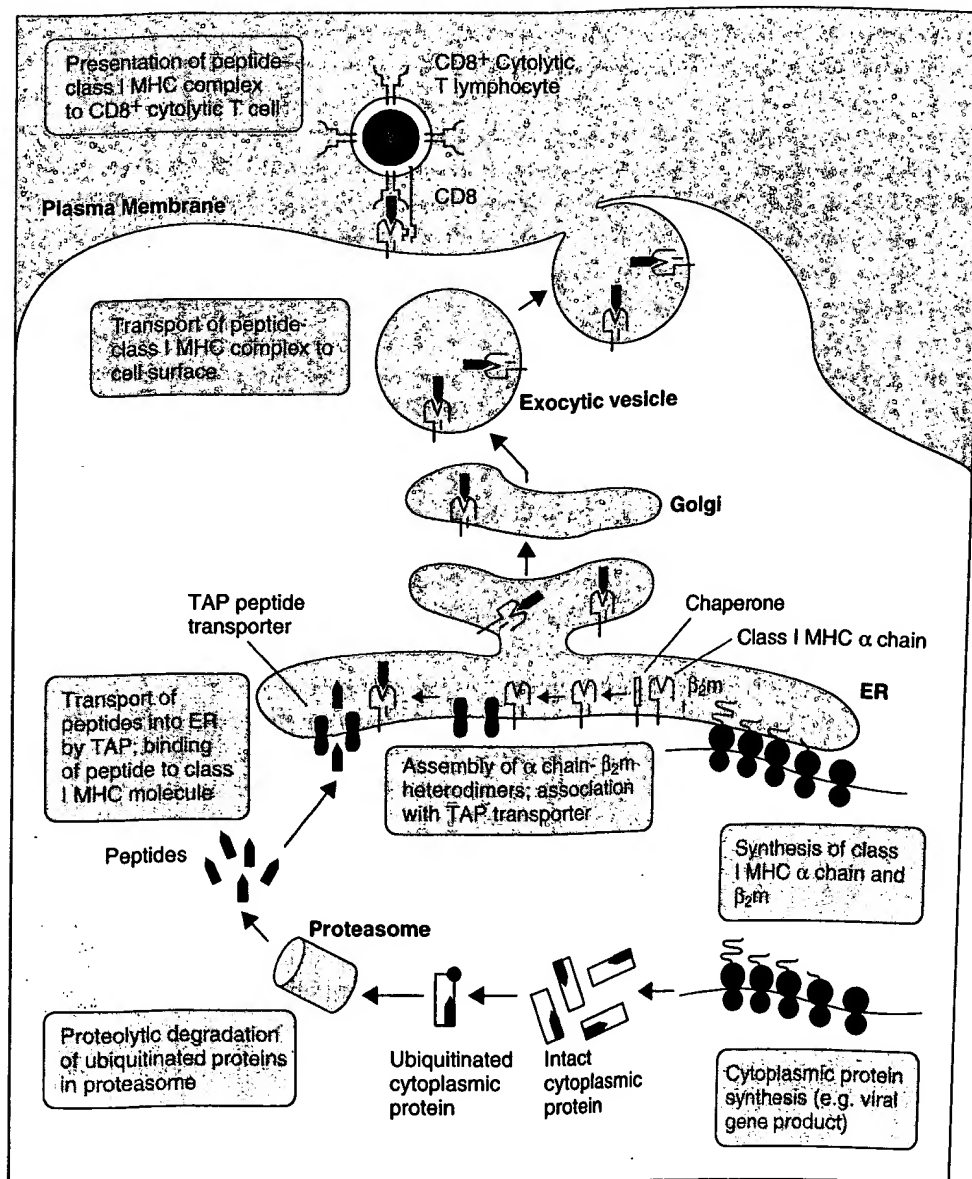


FIGURE 6-8. The class I major histocompatibility complex (MHC) pathway of antigen presentation. TAP, transporter associated with antigen presentation; ER, endoplasmic reticulum; β_2m , β_2 microglobulin.

Entry of Cytosolic Proteins Into the Class I-MHC Pathway of Antigen Presentation

The prerequisite for entry of a protein into the processing pathway leading to peptide-class I MHC association is simply location in the cytosol. Several lines of evidence support this.

1. If a viral protein, such as influenza nucleoprotein, or a protein like ovalbumin, is added in soluble form to a cell that expresses class I and class II MHC molecules, the antigen is internalized, processed, and presented only in association with class II MHC molecules. Such exogenously added antigens will be recognized by class II-restricted, antigen-specific CD4⁺ T cells but will not sensitize the APC to lysis by CD8⁺ T cells. On the other hand, if the gene encoding the viral protein or ovalbumin is transfected into the APCs so that the antigen is synthesized on polyribosomes in the cytosol, the cell becomes sensitive to lysis by specific class I-restricted CD8⁺ T cells (see Fig. 6-3).

2. If an antigen is introduced into the cytoplasm of a cell by making the plasma membrane transiently permeable to macromolecules or by membrane fusion of an APC with lipid vesicles containing the protein, the antigen is subsequently processed and peptides associate only with class I MHC molecules (see Fig. 6-3).

The significance of having cytosolic proteins enter the class I MHC pathway of antigen presentation lies in the fact that endogenously synthesized foreign or mutant proteins will be present in the cytosol, and therefore will target cells for lysis by CD8⁺ class I MHC-restricted CTL. For example, viruses encode RNA transcripts, which are translated into foreign proteins in the host cell cytoplasm. Therefore, peptides derived from viral protein antigens end up being displayed on class I MHC molecules on the surface of virally infected cells. This enables class I MHC-restricted CD8⁺ cytolytic T cells to recognize the virally infected cells and destroy them. Since virtually all nucleated cells express class I MHC, any virus-infected cell is susceptible to CTL-mediated lysis. Similarly, CTLs may be important in recognizing and killing cancer cells, which often express mutated genes or unmutated genes that are not expressed in normal adult cells (see Chapter 18). The products of such endogenous genes may be expressed in the cytosol. In addition, some intracellular microbes, such as mycobacteria, reside for long periods of time within phagocytic vesicles. It is possible that there will be some breakdown in the membrane barrier of these vesicles, resulting in the microbial proteins leaking into the cytoplasm, and thus gaining access to the class I MHC pathway of antigen presentation. Alternatively, there may be specific transport mechanisms that deliver proteins or peptides from these vesicles to the cytoplasm.

Processing of Cytosolic Antigens

The intracellular mechanisms that generate antigenic peptides which bind to class I MHC molecules are very different from the mechanisms described earlier for peptide-class II MHC molecule associations. This is evident from the observations that the agents that raise endosomal and lysosomal pH, or directly inhibit endosomal proteases, block class II- but not class I-associated antigen presentation.

Peptides that bind to class I MHC molecules are proteolytically generated in the cytoplasm prior to entry into the exocytic pathway that delivers the peptide-MHC protein complex to the cell surface. This conclusion is supported by a variety of experimental observations.

1. A cell infected with a virus becomes sensitized to lysis by virus-specific CTLs; this is because the cell displays peptides derived from viral proteins in association with class I MHC molecules on the cell surface. Some of these proteins, such as influenza nucleoprotein, are neither membrane bound nor secreted, i.e., they do not gain access to exocytic pathways in their intact form. Furthermore, the genes encoding viral membrane proteins can be altered to eliminate the membrane insertion sequences. When these genes are transfected into cells, the encoded proteins cannot gain access to the ER and exocytic pathway, yet peptides from these proteins are still presented to CD8⁺ CTLs.

2. When peptide epitopes for CTL recognition are synthesized directly in the cytoplasm of a cell as products of transfected minigenes, the cell becomes sensitized for lysis. This implies that peptides generated in the cytoplasm have direct access to the exocytic pathway for cell surface expression of class I MHC molecules.

*A major mechanism for the generation of peptides from cytosolic protein antigens is proteolysis in the proteasome, a large multiprotein complex with a broad range of proteolytic activity that is found in the cytoplasm of most cells. A 700 kD form of proteasome appears as a cylinder composed of a stacked array of four inner and four outer rings, with each ring composed of seven distinct subunits. The subunits of the inner rings are the catalytic sites for proteolysis. A larger, 1500 kD proteasome is likely to be most important *in vivo* and is composed of the 700 kD structure plus several additional subunits that regulate proteolytic activity. Two catalytic subunits present in many 1500 kD proteasomes, called LMP2 and LMP7, are encoded by genes in the MHC (see Chapter 5). Both LMP2 and LMP7 expression are upregulated by IFN- γ , leading to an increase in the number of proteasomes containing these subunits. The proteasome performs a basic housekeeping function in cells by degrading many different cytoplasmic proteins. For example, NF- κ B activation is dependent on proteasomal degradation of I κ B (see Box 4-4, Chap-*

ter 4). Proteins are targeted for proteasomal degradation by covalent linkage of several copies of a small polypeptide called ubiquitin. This process of polyubiquitination requires adenosine triphosphate (ATP) and a variety of enzymes. Several lines of evidence suggest that the proteasome, and probably ubiquitination, are involved in antigen processing for the class I MHC pathway of antigen presentation.

1. In some experimental situations, inhibition of the enzymes required for ubiquitination also inhibits the presentation of cytoplasmic proteins to class I MHC-restricted T cells specific for a peptide epitope of that protein.

2. Modification of proteins by attachment of an N-terminal sequence which is recognized by ubiquitin-conjugating enzymes leads to enhanced ubiquitination and more rapid class I MHC-associated presentation of peptides derived from those proteins.

3. Specific inhibitors of proteasomal function, such as peptide aldehydes, block presentation of a cytoplasmic protein to class I MHC-restricted T cells specific for a peptide epitope of that protein.

4. Proteasomes typically generate peptides between five and 11 amino acids long, which includes the lengths that best fit the peptide-binding clefts of class I MHC molecules.

5. The specificity of proteolysis by LMP-2- and LMP-7-containing proteasomes from IFN- γ -treated cells favors the generation of peptides with C-terminal basic or hydrophobic amino acid residues, which are typical of many class I MHC-binding peptides.

There are many examples of protein antigens that apparently do not require ubiquitination or proteasomes in order to be presented by the class I MHC pathway. In some cases this may reflect the fact that other, less well-defined mechanisms of cytoplasmic proteolysis exist. In addition, some class I MHC-binding peptides may be generated by proteolytic enzymes resident in the ER. For example, peptides from secretory proteins with hydrophobic signal sequences are often found associated with class I MHC molecules. These proteins are targeted directly to the ER during translation and therefore may bypass cytoplasmic degradation.

Delivery of Peptides From Cytoplasm to the ER

Class I MHC molecules are assembled in the ER, and this process is dependent on peptides. Since peptides generated in the cytosol are presented by class I MHC molecules, a mechanism must exist for delivery of cytosolic peptides into the ER. The initial insights into this mechanism came from studies of cell lines that are defective in assembling and displaying peptide-class I MHC complexes on their surfaces. The mutations responsible for this defect turned out to involve two genes in the MHC, which are homologous to a fam-

ily of genes that encode proteins that mediate ATP-dependent transport of low molecular weight compounds across intracellular membranes. The two genes in the MHC that belong to this family encode proteins called transporter associated with antigen presentation-1 or TAP-1, and TAP-2. TAP-1 and TAP-2 form heterodimers, which are localized in the ER and *cis*-Golgi (Fig. 6-8). In this location they mediate the active, ATP-dependent transport of peptides from the cytosol into the ER lumen. Although the TAP heterodimer has a broad range of specificities, it optimally transports peptides ranging from eight to 12 amino acid residues long and therefore delivers to the ER peptides of the right size for binding to class I MHC molecules. Mice with targeted disruptions of the genes encoding TAP-1 or TAP-2 show defects in class I MHC expression and cannot effectively present proteins to class I MHC-restricted T cells. Rare examples of human TAP-2 gene mutations have also been identified, and predictably, the patients carrying these mutant genes also show defective class I MHC-associated antigen presentation.

Assembly and Surface Expression of Peptide-Class I MHC Complexes

The actual assembly and surface expression of stable class I MHC molecules require the presence of peptides. A variety of experimental data have indicated a particular sequence of events in assembly and expression of peptide-class I MHC complexes:

1. The class I MHC α chain and β_2 microglobulin are synthesized on the rough ER and transported into the smooth ER as separate polypeptide chains.

2. The α chain associates with molecular chaperones, which prevent degradation and promote proper folding of the protein. Two chaperones that are known to associate with the α chain in the ER are BiP, a member of the heat shock protein family, and calnexin.

3. β_2 microglobulin binds to partially or completely folded α chain and the chaperones dissociate. These newly formed α chain- β_2 microglobulin dimers are unstable and cannot be transported efficiently out of the ER.

4. The α chain- β_2 microglobulin dimers move to and become physically associated with the luminal aspects of the TAP proteins within the ER. This close association ensures that peptides transported into the ER by the TAP bind to the associated empty class I MHC molecules. It is also possible that the TAP association promotes further folding of the α chain and β_2 microglobulin.

5. Peptide binding to the class I molecule greatly enhances its stability and causes its release from the TAP protein.

6. Stable peptide-class I MHC complexes now move through the Golgi, where the MHC molecules

undergo further carbohydrate modification, and then they are transported to the cell surface by exocytic vesicles. Surface complexes can now be recognized by CD8⁺ T cells.

The requirement for peptides in class I MHC assembly has been clearly shown by analysis of TAP-deficient cells (either mutant cell lines or cells from TAP-1 gene knockout mice), which express significantly reduced levels of surface class I MHC (Fig. 6-9). Since TAP delivers peptides to the ER, these findings suggest that peptides in the ER are required for class I MHC assembly. Those class I MHC molecules that do get expressed in TAP-deficient cells have bound peptides that are mostly derived from signal sequences of proteins destined for secretion or membrane expression. These signal sequences are cleaved off and degraded to peptides within the ER during translation, without a requirement for TAP. There are two reasons why peptides transported into the ER preferentially bind to class I and not class II MHC molecules. First, as we have discussed, newly formed class I MHC molecules are bound to the luminal aspect of the TAP complex. Second, as mentioned previously, in the ER the class II MHC-peptide-binding cleft is blocked by the invariant chain.

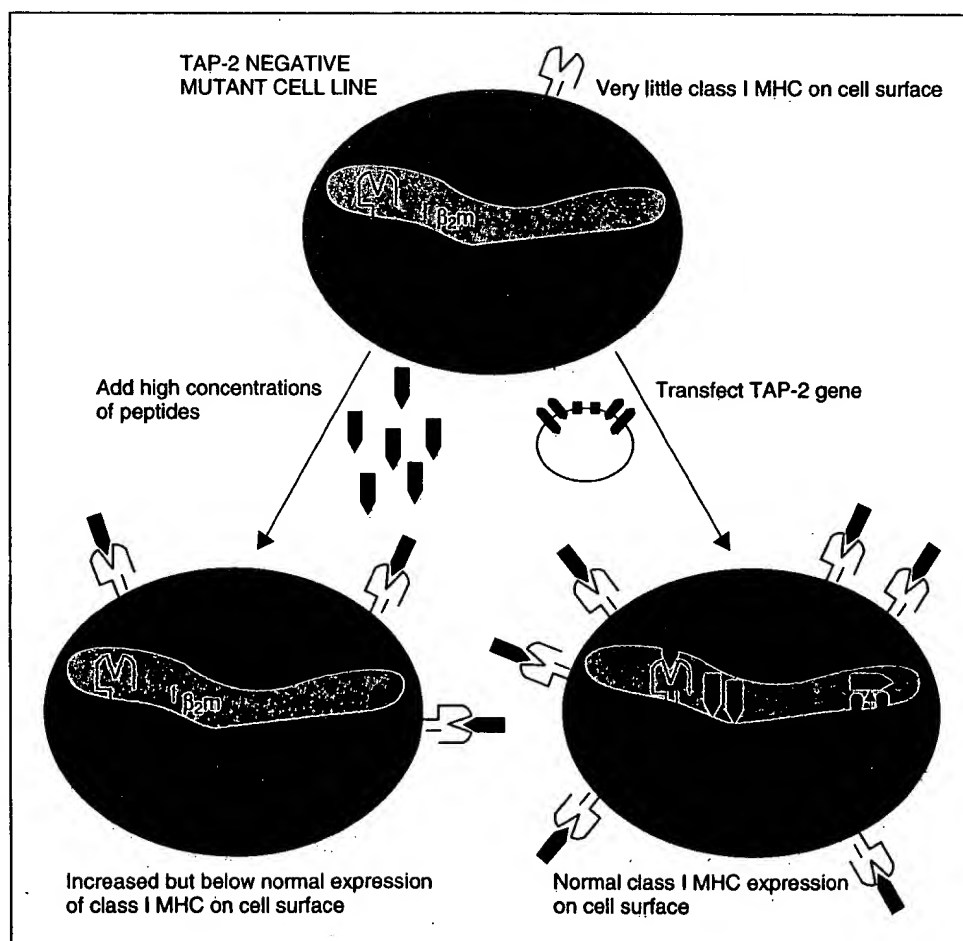
The sequence of events in class I MHC mole-

cule assembly which we have discussed ensures that only properly folded, peptide-loaded class I MHC molecules are displayed for T cell surveillance. A few empty class I MHC complexes do make it out to the cell surface, but these are unstable and rapidly dissociate. It is, of course, likely that there are other steps involved in this pathway that are not yet resolved, and it is also possible that alternate pathways may exist. Nonetheless, the effects of mutations and inhibitors of this pathway, as discussed, indicate that it is critical for normal immune function. Furthermore, the importance of this pathway to anti-viral immunity is demonstrated by the evolution of viral mechanisms that interfere with it. For example, herpes simplex virus produces a protein, called ICP47, that effectively plugs up the TAP pore through which peptides are delivered to the ER and thus prevents presentation of viral antigens to T cells (see Chapter 16).

PHYSIOLOGIC SIGNIFICANCE OF MHC-ASSOCIATED ANTIGEN PRESENTATION

So far we have discussed the specificity of CD4⁺ and CD8⁺ T lymphocytes for MHC-associated foreign protein antigens and the mechanisms by which complexes of peptides and MHC molecules

FIGURE 6-9. TAP gene products are required for assembly and cell surface expression of peptide-class I major histocompatibility (MHC) complexes. A cell line with a nonfunctional TAP-2 gene expresses very few surface class I MHC molecules. The peptides bound to these few surface class I MHC molecules are predominantly derived from the signal sequences of membrane or secreted proteins. The addition of high doses of peptides can induce some class I MHC molecule assembly and expression. In this case, it is not known whether the assembly of the peptide-class I complexes occurs at the cell surface or intracellularly. When a functional TAP-2 gene is transfected into the cell line, normal assembly and expression of peptide-class I MHC molecules are restored.



are produced. There are several fundamental properties of T cell-mediated immune responses that are consequences of the fact that T cells only recognize MHC-associated antigens. In this section, we will consider the impact of MHC-associated antigen presentation on the role that T cells play in protective immunity, the nature of T cell responses to different antigens, and the limitations of what T cells will recognize in protein antigens.

T Cell Surveillance for Foreign Antigens

As we discussed throughout this chapter, both the class I and class II MHC pathways of antigen presentation sample pools of predominantly normal self proteins for display to the T cell repertoire, which surveys these samples for the rare foreign or mutant peptide. The recent advances in our understanding of how peptide-MHC complexes are formed confirm that MHC molecules are scaffolds for peptide display to the immune system and that antigen processing pathways have evolved to sample both extracellular and intracellular proteins in order to supply the peptides. The specialized class II MHC-expressing APCs have various characteristics, such as the phagocytic activity of macrophages, the high-affinity Ig antigen receptors on B cells, and the long cytoplasmic processes of dendritic cells, which enable them to encounter the full range of possible extracellular protein antigens. The convergence of the endocytic pathways in these cells with the exocytic pathway of class II MHC expression ensures that peptides derived from these extracellular antigens will be displayed on the cell surface for possible recognition by CD4⁺ T cells. The widespread expression of class I MHC in nucleated cells, and the pathway of peptide loading of class I MHC molecules which is linked to a ubiquitous mechanism for degrading cellular proteins, ensures that peptides from virtually any intracellular protein will be displayed for possible recognition by CD8⁺ T cells. Superimposed on this system of antigen presentation is a sensitive system of T cell surveillance of the displayed peptides, which is based on continuous recirculation of T cells to sites of APCs throughout the body, and the exquisite sensitivity of T cells, allowing them to respond to small numbers of peptide-MHC complexes. Thus, the paradox that antigen presentation mechanisms overwhelmingly display normal self peptides is actually fundamental to the ability of the immune system to find rare foreign protein antigens.

The Nature of T Cell Responses

Based on our understanding of antigen presentation to T cells, we can now explain other physiologic consequences of MHC-restricted antigen recognition that were introduced in Chapter 5.

1. Because T cells recognize only MHC-associated peptide antigens, they can respond only to

antigens associated with other cells (the APCs) and are unresponsive to soluble or circulating proteins. *This unique specificity for cell-bound antigens is essential for the functions of T lymphocytes, which are largely mediated by cell-cell interactions and by cytokines that act at short distances.* For instance, helper T cells activate B cells and macrophages. Not surprisingly, B lymphocytes and macrophages are two of the principal cell types that express class II MHC genes, function as APCs for CD4⁺ helper T cells, and focus helper T cell effects to their immediate vicinity. Similarly, CTLs can lyse any nucleated cell producing a foreign antigen, and all nucleated cells express class I MHC molecules, which are the restricting elements for antigen recognition by CD8⁺ CTLs.

2. The triaging of endosomal versus cytoplasmic proteins to class II or class I MHC pathways of antigen presentation determines which subsets of T cells will respond to antigens found in those two pools of proteins (Fig. 6-10). Extracellular antigens usually end up in the endosomal pool and activate class II-restricted CD4⁺ T cells. These cells function as helpers to stimulate effector mechanisms such as antibodies and phagocytes that serve to eliminate extracellular antigens. Conversely, endogenously synthesized antigens are present in the cytoplasmic pool of proteins and usually activate class I-restricted CD8⁺ CTLs. These lymphocytes lyse cells producing intracellular antigens. *Thus, antigens from microbes that reside in different locations selectively stimulate the T cell populations that are most effective at eliminating that type of microbe.*

Immunogenicity of Protein Antigens

MHC molecules may determine the immunogenicity of protein antigens in two related ways:

1. *The immunodominant epitopes of complex proteins are often the peptides that bind most avidly to MHC molecules.* If an individual is immunized with a multideterminant protein antigen, in many instances the majority of the responding T cells are specific for one or a few linear amino acid sequences of the antigen. These are called the "immunodominant" determinants or epitopes. For instance, in H-2^k mice immunized with hen egg lysozyme (HEL), more than half the HEL-specific T cells are specific for the epitope formed by residues 46-61 of HEL in association with the I-A^k but not the I-E^k molecule. This is because HEL(46-61) binds to I-A^k better than do other HEL peptides, and does not bind to I-E^k. However, it is not yet known exactly which structural features of a peptide determine immunodominance. As mentioned earlier, for class I-restricted antigen presentation, immunodominant peptides are required to have amino acid residues whose side chains fit into pockets of the MHC molecule-peptide-binding cleft. Common features of immunodominant peptides for class II MHC-restricted antigen presenta-

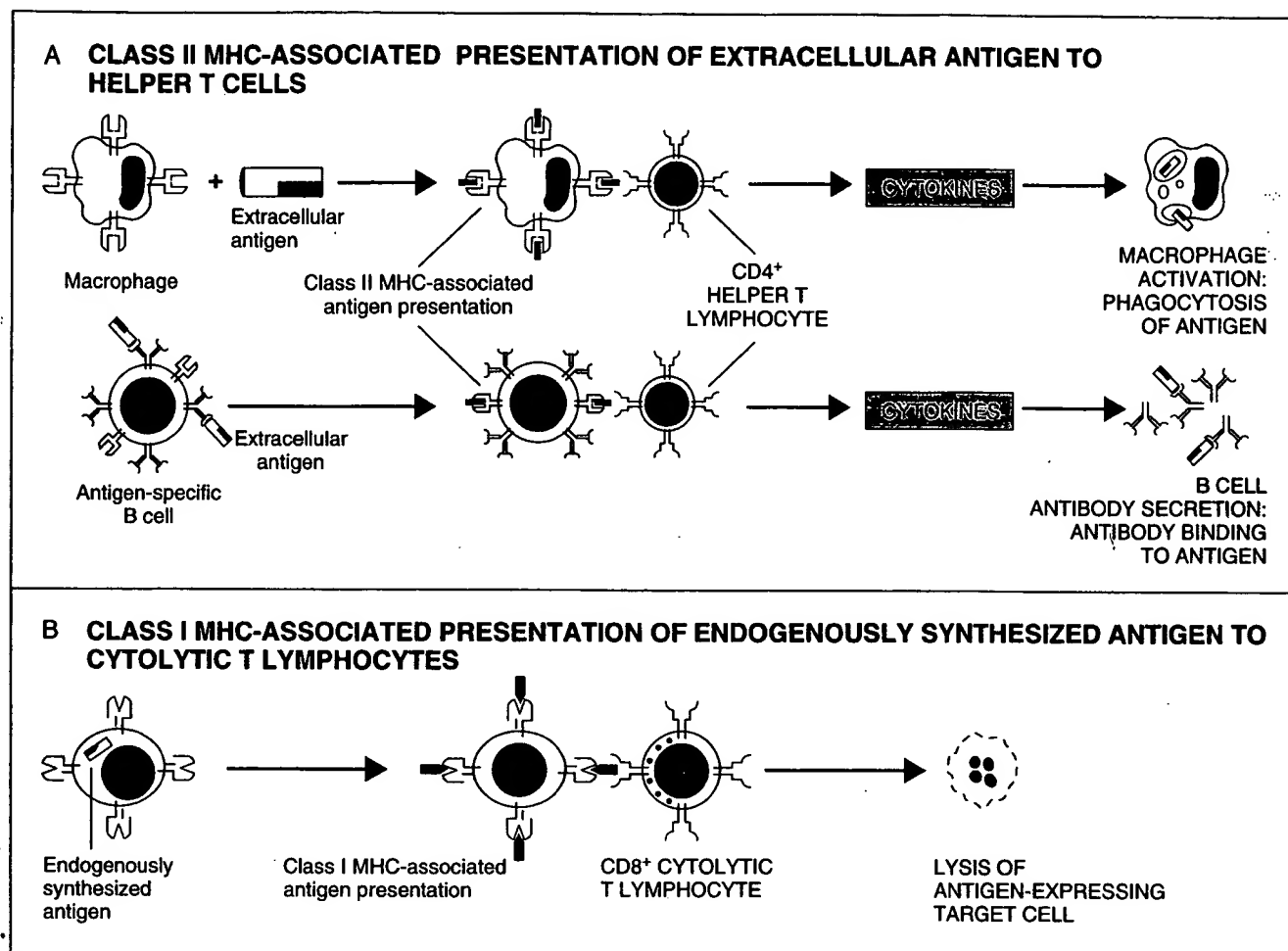


FIGURE 6-10. Presentation of exogenous and endogenous protein antigens to different subsets of T cells. MHC, major histocompatibility complex.

tion are less well defined. The question is an important one because an understanding of these features may permit the efficient manipulation of the immune system with synthetic peptides. An obvious application of such knowledge is the design of vaccines. For example, a protein encoded by a viral gene could be analyzed for the presence of amino acid sequences that would form a typical immunodominant secondary structure capable of binding to MHC molecules with high affinity. Vaccines composed of synthetic peptides mimicking this region of the protein theoretically would be effective in eliciting T cell responses against the viral peptide expressed on an infected cell, thereby establishing protective immunity against the virus.

2. *The expression of particular class II MHC alleles in an individual determines the ability of that individual to respond to particular antigens.* The phenomenon of immune response (Ir) gene-controlled immune responsiveness was mentioned in Chapter 5. We now know that Ir genes that control antibody responses are class II MHC genes. They influence immune responsiveness in part because various allelic class II MHC molecules differ in their ability to bind different antigenic peptides and,

therefore, to stimulate specific helper T cells. For instance, H-2^k mice are responders to HEL(46-61), but H-2^d mice are non-responders to this epitope. Equilibrium dialysis experiments have shown that HEL(46-61) binds to I-A^k but not to I-A^d molecules. A possible molecular basis for this difference in MHC association is suggested from the model of the class II molecule and the known amino acid sequences of I-A^k and I-A^d proteins. If the HEL(46-61) peptide is hypothetically placed in the predicted binding cleft of the I-A^k molecule, charged residues of the HEL peptide become aligned with oppositely charged residues of the MHC molecule. This would presumably stabilize the bimolecular interaction. In contrast, the I-A^d molecule has different amino acids in the binding cleft that would result in the aligning of like-charged residues with the HEL peptide. Therefore, HEL(46-61) would not bind to or be presented in association with I-A^d, and the H-2^d mouse would be a non-responder. Similar results have been obtained with numerous other peptides. MHC-linked immune responsiveness may also be important in humans. For instance, Caucasians who are homozygous for an extended HLA haplotype containing HLA-B8,DR3,

DQw2a are low responders to hepatitis B virus surface antigen. Individuals who are heterozygous at this locus are high responders, presumably because the other alleles contain one or more HLA gene that confers responsiveness to this antigen. Thus, HLA typing may prove to be valuable for predicting the success of vaccination. These findings support the **determinant selection model** of MHC-linked immune responses. This model, which was proposed many years before the demonstration of peptide-MHC binding, states that the products of MHC genes in each individual select which determinants of protein antigens will be immunogenic in that individual. We now understand the structural basis of determinant selection and Ir gene function in antigen presentation. Most Ir gene phenomena have been studied by measuring helper T cell function, but the same principles apply to CTLs. Individuals with certain MHC alleles may be incapable of generating CTLs against some viruses. In this situation, of course, the Ir genes may map to one of the class I MHC loci.

Although these concepts are based largely on studies with simple peptide antigens and inbred strains of mice, they are also relevant to the understanding of immune responses to complex multideterminant protein antigens in outbred species. It is likely that all individuals will express at least one MHC molecule capable of binding at least one determinant of a complex protein, so that all individuals will be responders to such antigens. As stated in Chapter 5, this may be the evolutionary pressure for maintaining MHC polymorphism.

This discussion of the influence of MHC gene products on the immunogenicity of protein antigens has focused on antigen presentation and has not considered the role of the T cells. We have mentioned earlier that the exquisite specificity and diversity of antigen recognition are attributable to antigen receptors on T cells. MHC-linked immune responsiveness is also dependent, in part, on the presence and absence of specific T cells. In fact, some peptides may bind to MHC molecules in a particular inbred mouse strain but do not activate T cells in that strain. It is likely that these mice lack T cells capable of recognizing the particular peptide-MHC complexes. *Thus, Ir genes may function by determining antigen presentation or by shaping the repertoire of antigen-responsive T cells.* The development of the T cell repertoire and the role of the MHC in T cell maturation are discussed in Chapter 8.

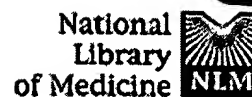
SUMMARY

T cells recognize antigens only on the surface of accessory cells in association with the products of self MHC genes. CD4⁺ helper T lymphocytes recognize antigens in association with class II MHC

gene products (class II MHC-restricted recognition), and CD8⁺ CTLs recognize antigens in association with class I gene products (class I MHC-restricted recognition). Antigen processing consists of the introduction of protein antigens into APCs, the proteolytic degradation of these proteins into peptides, the binding of peptides to newly assembled MHC molecules, and the display of the peptide-MHC complexes on the APC surface for potential recognition by T cells. Antigen-processing pathways in APCs utilize basic cellular proteolytic mechanisms, which also operate independent of the immune system. Both extracellular and intracellular proteins are sampled by these antigen-processing pathways, and peptides derived from both normal self proteins and foreign proteins are displayed by MHC molecules for surveillance by T lymphocytes. Specialized APCs, including macrophages, B lymphocytes, and dendritic cells, internalize extracellular proteins into endosomes for processing by the class II MHC pathway. These proteins are proteolytically cleaved by enzymes that function at acidic pH in vesicles of the endosomal pathway. Newly synthesized class II MHC heterodimers associate with the invariant chain and are directed from the ER to the endosomal vesicles, where the invariant chain is proteolytically cleaved, and a small peptide remnant of the invariant chain is removed from the peptide binding cleft of the MHC molecule by the DM molecules. The peptides generated from extracellular proteins then bind to the class II MHC molecule, and the trimeric complex (class II MHC α and β chains and peptide) moves to the surface of the cell. Cytosolic proteins, usually synthesized in the cells, such as viral proteins, enter the class I MHC pathway of antigen presentation. The proteasome is a cytoplasmic multiprotein complex which proteolytically degrades ubiquitinated cytoplasmic proteins and probably generates a large part of the peptides destined for display by class I MHC molecules. Peptides are delivered from the cytoplasm to the ER by the TAP molecules. Newly formed class I MHC dimers in the ER associate with and bind peptides delivered by TAP. Peptide binding stabilizes class I MHC molecules and permits their movement out of the ER, through the Golgi, to the cell surface. These pathways of MHC-restricted antigen presentation ensure that most of the body's proteins are screened for the possible presence of foreign antigens. The pathways also ensure that proteins from extracellular microbes are likely to generate peptides bound to class II MHC molecules for recognition by CD4⁺ helper T cells, while proteins encoded by intracellular microbes generate peptides bound to class I MHC molecules for recognition by CD8⁺ CTLs. The immunogenicity of microbial proteins depends on the ability of antigen-processing pathways to generate peptides from these proteins which bind to self MHC molecules.

Selected Readings

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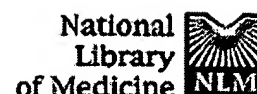
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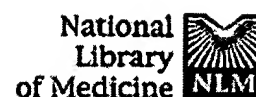
We have studied the binding of M467, an IgA murine myeloma protein, to flagellin from seven species of Salmonella. It was found that M467 was reacting with antigenic determinants that were common to all the flagellins studied. These determinants were not related to serotypic antigens. Electronmicrographs of unreduced M467 showed a variety of polymeric species bound to flagella in a manner that could produce immobilization as well as agglutination and precipitation through cross-linking of antigenic determinants. Immunodiffusion in agar gel revealed that M467 was recognizing more than one group of peptide determinants on the flagellins studied. Passive hemagglutination inhibition and a solid phase radioimmunoassay provided evidence that there were differences in binding avidities between M467 and the various Salmonella flagellins studied. It was concluded that M467 is binding more than one specific group of antigenic peptide determinants on flagellin molecules. Flagellin from four of the seven species of Salmonella studied were deficient in one or more of these determinants.

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